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IMMUNOTHERAPY OF MALIGNANT AND AUTOIMMUNE DISORDERS IN DOMESTIC ANIMALS USING NAKED ANTIBODIES, IMMUNOCONJUGATES AND FUSION PROTEINS

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to immunotherapeutic methods for treating B-cell and T-cell, myeloid, mast-cell and plasma-cell disorders in domestic animals, particularly in companion animals such as dogs, cats, and horses. In particular, this invention is directed to methods for treating B-cell and T-cell, myeloid, mast cell, and plasma-cell disorders by administering comparatively low doses of naked antibodies against antigens associated with these cell types, which are equivalent to the respective normal lineage and non-lineage antigens present in similar normal cells of humans, by antibody given alone, by antibody combinations in which each antibody binds to a different target antigen or antigen epitope, or by administering an immunoconjugate in which at least one antibody component is conjugated to a therapeutic agent. The present invention also is directed to multimodal therapeutic methods in which naked antibody or immunoconjugate administration is supplemented with chemotherapy, radiotherapy, cytokines, or by administration of therapeutic proteins, such as antibody fusion proteins. The present invention also contemplates the combination of such antibodies and antibody conjugates with lymphoma-, leukemia-, or myeloma-specific antibodies, which bind more selectively to such malignant cells than to their normal cell counterparts. Examples of such lymphoma-specific antibodies are those described, in U.S. patent 5,169,775, issued to Steplewski *et al.*, which are preferentially reactive with canine lymphoma cells and insignificantly reactive with normal lymphocytes, and do not react with DR antigens of the dog, which are equivalent with HLA-DR antigens in humans and Ia antigens in humans and mice. Although the antibodies of this invention react with both normal and malignant cells of domestic animals, administration of these antibodies in animals having malignancies of these cell populations results in tumor responses and only minimal side effects because of the concomitant transient depletion of target normal cells.

Background

[0002] B- and T-cell lymphomas and leukemias, such as the B-cell subtype of non-Hodgkin's lymphoma (NHL) and T-cell leukemias, are significant contributors to cancer mortality in domestic animals and are on the increase, particularly in companion animals such as dogs and cats. Significant similarities between human and canine NHL have been reported. See, for example, Fournel-Fleury, *et al., J. Comp. Pathol.*, 117(1):35-59 (1997); Ruslander *et al., In Vivo*, 11(2):169-72 (1997). In particular, fine-needle aspirates from 21 dogs with peripheral lymphadenopathy (18 with lymphoma and three with lymph node hyperplasia) showed that 14 of the lymphomas were B-cell lymphomas. Caniatti *et al., Vet. Pathol.*, 33(2):204-12 (1996). Ruslander *et al.* reported that 76% (134/175) of dogs with lymphoma were determined to be derived from B-cells. Similarly, Day *et al.* reported in a study based on eight cats that the clinical, histological and immunophenotypic findings in cats were identical with those of NHL in humans. *J. Comp. Pathol.*, 120(2):155-67 (1999).

[0003] The response of B-cell and T-cell malignancies to various forms of treatment is mixed in both humans and animals. For example, in humans in cases in which adequate clinical staging of non-Hodgkin's lymphoma is possible, field radiation therapy can provide satisfactory treatment. Still, about one-half of the patients die from the disease. In dogs, standard treatment involves chemotherapy with a combination of vincristine, cyclophosphamide, prednisolone, doxorubicin, and L-asparaginase.

[0004] The majority of chronic lymphocytic leukemias in humans are of B-cell lineage. Freedman, *Hematol. Oncol. Clin. North Am. 4*:405 (1990). This type of B-cell malignancy is the most common leukemia in the Western world. Goodman *et al., Leukemia and Lymphoma 22*:1 (1996). The natural history of chronic lymphocytic leukemia falls into several phases. In the early phase, chronic lymphocytic leukemia is an indolent disease, characterized by the accumulation of small mature functionally-incompetent malignant B-cells having a lengthened life span. Eventually, the doubling time of the malignant B-cells decreases and patients become increasingly symptomatic. While treatment can provide symptomatic relief, the overall survival of the patients is only minimally affected. The late stages of chronic lymphocytic leukemia are characterized by significant anemia and/or thrombocytopenia. At this point, the median survival is less than two years. Foon *et al., Annals Int. Medicine*

113:525 (1990). Due to the very low rate of cellular proliferation, chronic lymphocytic leukemia is resistant to treatment.

[0005] B-cell leukemias have also been identified in canines. Nakaichi *et al.*, *J. Vet. Med. Sci.* 58(5):469-71 (1996). Canines and felines also are afflicted with numerous lymphoproliferative disorders. In one study of 175 dogs with lymphoma, 134 were determined to be derived from B-cells and 38 were derived from T-cells. Ruslander *et al.*, *In Vivo* 11(2):169-172 (1997). Day *et al.* reported on cases of T-cell rich, B-cell lymphoma in cats. Treatment options for domestic animals generally are limited to chemotherapy, however.

[0006] Canine lymphoma has been found to be similar to human non-Hodgkin's lymphoma in pathological types, response to the same chemotherapeutic agents, correlation of immunophenotyping of cell surface markers to histological classification and chemosensitivity, and distribution of B, T, and non-T, non-B cell lymphomas (Macewen *et al.*, *J. Am. Vet. Med. Assoc.* 178:1178, (1981); Applebaum *et al.*, *Hematol. Oncol.* 2:151, (1984); Carter *et al. Canad. J. Vet. Res.* 50:154, (1986)). It is thus reasonable to postulate that equivalent markers on B- and T-cells might serve as useful targets for therapeutic approaches, as found in humans. Similar markers on myeloid-, plasma-, and mast-cells, which transform to malignancies in domestic animals, as in humans, may also serve as targets for therapeutic agents. In contrast to humans, however, mastocytomas are the most frequent neoplasm in dogs, and thus may have a different pathogenesis and etiology than in humans. Although lineage and non-lineage antigens associated with these cell types will result in depletion of these cells when specific antibodies are given, there will be an advantageous suppression of the malignant cells without intolerable side effects to the host animals by immunotherapeutic use of these antibodies. Thus, we have discovered that an absolute or even high specificity of such antibodies for malignant as compared to normal cells is not required for achieving therapeutic responses in these domestic animals. Indeed, the binding of such antibodies to the malignant cells can be less than double that observed in their normal cell counterparts, thus being markedly different from the invention of Steplewski *et al.*, *infra*.

[0007] Traditional methods of treating B-cell and T-cell malignancies, including chemotherapy and radiotherapy, have limited utility due to toxic side effects. The use of monoclonal antibodies to direct radionuclides, toxins, or other therapeutic agents offers the

possibility that such agents can be delivered selectively to tumor sites, thus limiting toxicity to normal tissues.

[0008] Antibodies against the CD20 antigen have been investigated for the therapy of B-cell lymphomas in humans. For example, a chimeric anti-CD20 antibody, designated as "IDEC-C2B8," has activity against B-cell lymphomas when provided as unconjugated antibodies at repeated injections of doses exceeding 500 mg per injection. Maloney *et al., Blood* 84:2457 (1994); Longo, *Curr. Opin. Oncol. 8*:353 (1996). About 50 percent of non-Hodgkin's patients, having the low-grade indolent form, treated with this regimen showed responses. Therapeutic responses have also been obtained using ^{131}I-labeled B1 anti-CD-20 murine monoclonal antibody when provided as repeated doses exceeding 600 mg per injection. Kaminski *et al., N. Engl. J. Med. 329*:459 (1993); Press *et al., N. Engl. J. Med. 329*:1219 (1993); Press *et al., Lancet 346*:336 (1995). However, these antibodies, whether provided as unconjugated forms or radiolabeled forms, have shown less impressive responses in patients with the more prevalent and lethal form of B-cell lymphoma, the intermediate or aggressive type.

[0009] A need exists to develop an immunotherapy for B-cell and T-cell malignancies, as well as myeloid, mast-cell, and plasma-cell (myeloma) malignancies, in domestic animals, particularly in companion animals such as dogs and cats, that allows repeated administration of comparatively low doses of an antibody, and that is not limited by the necessity of adding a toxic agent for achieving a therapeutic response of significant duration.

[0010] Autoimmune diseases are a class of diseases associated with a B-cell or T-cell disorder. Examples include immune-mediated autoimmune hemolytic anemia, canine granulomatous meningoencephalitis, rheumatoid arthritis, chronic superficial keratitis, systemic lupus erythematosus, bullous pemphigoid, pemphigus, and thrombocytopenias. The most common treatments are corticosteroids and cytotoxic drugs, which can be very toxic. These drugs also suppress the entire immune system, can result in serious infection, and have adverse affects on the liver and kidneys. Other therapeutics that have been used to treat Class III autoimmune diseases to date have been directed against T-cells and macrophages. A need also exists for more effective methods of treating autoimmune diseases, particularly Class III autoimmune diseases.

SUMMARY OF THE INVENTION

[0001] The present invention provides methods of treating a B-cell, T-cell, myeloid, mast-cell, or plasma-cell disorder in a domestic animal, particularly in a companion animal such as a dog or cat. The method entails the administration of antibodies directed against antigen determinants found on cells of these malignant neoplasms or which generate the autoimmune disease cells or antibodies. These antibodies recognize antigens that are equivalent to similar lineage and non-lineage antigens expressed by normal human B-, T-, myeloid-, plasma-, and mast-cells. A complete listing of normal human lineage and non-lineage antigens can be found in the 5th CD Leucocyte Typing Workshop (1995), the contents of which are incorporated herein in their entirety by reference. Animal equivalents of these antigens, which may vary by species, are readily identifiable and their use is preferred in methods according to the present invention.

[0002] These antibodies can be either given by themselves as unconjugated immunoglobulins, in antibody combinations, or as antibody conjugates with isotopes, drugs, or other therapeutic modalities, or still other applications of such monoclonal antibodies in combination with other therapy modalities, such as chemotherapy, radiation therapy, cytokine therapy, or bispecific antibody fusion proteins involving other therapeutic agents or multiple targets. Many of these antigen targets on suitable malignant cells or normal hematopoietic or tissue cells involved in generating automimmune disease are similar to or counterparts of equivalent structures on human cells, such as B-cell complex, T-cell, myeloid, mast-cell, plasma cell, and HLA-DR antigens.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

1. Overview

Immunoconjugates as well as unconjugated or so-called "naked" antibodies to any known antigen that is characteristic of a B-cell, T-cell, myeloid-, plasma-, or mast-cell disorder can be used in accordance with the present invention to treat B-cell, T-cell, myeloid-, plasma-, or mast-cell disorder in domestic animals, particularly in dogs and cats. Preferred B-cell antigens include those equivalent to human CD19, CD20, CD21, CD22, CD52, CD74, and CD5 antigens. Preferred T-cell antigens include those equivalent to human CD4, CD8 and CD25 (the IL-2 receptor) antigens. An equivalent to HLA-DR antigen can be used in treatment of both B-cell and T-cell disorders. Particularly preferred B-cell antigens are those

equivalent to human CD19, CD20, CD21 and HLA-DR) antigens. Particularly preferred T-cell antigens are those equivalent to human CD4, CD8 and CD25 antigens.

[0001] The human CD33, CD14 and CD15 antigens are markers for myeloid leukemias, and antibodies to equivalent antigens in domestic animals are preferred in the treatment of such disorders in domestic animals. MUC1 and Ia (HLA-DR) antigens are markers for myeloma and other plasma-cell disorders, and their equivalents in domestic animals can be used to treat corresponding disorders in these animals.

[0002] The foregoing are exemplary, and not all-inclusive for lineage and non-lineage markers of B-, T-, myeloid-, plasma-, and mast-cells. Antibodies to equivalent antigens in domestic animals to such lineage and non-lineage antigens associated with these cell types can be used to treat such disorders in these domestic species. It has been reported that some of these antigens present on human cells are also detected in domestic animals when using suitable antibodies against the human cell antigens (Jacobsen *et al.*, *Vet. Immunol. Immunopathol.* 39:461, (1993); Cobbold and Metcalfe, *Tissue Antigens* 43:137, (1994); Moore *et al.*, *Tissue Antigens* 40:75, (1992); Greenlee *et al.*, *Vet. Immunol. Immunopathol.* 15:285, (1987); Aasted *et al.*, *Vet. Immunol. Immunopathol.* 19:31, (1988); Caniatti *et al.*, *Vet. Pathol.* 33:204, (1996); Darbes *et al.*, *J. Vet. Diagn. Invest.* 9:94, (1997); Grindem, *Vet. Clin. N. Amer.* 26:1043, (1996); Fournel-Fleury *et al.*, *J. Comp. Pathol.* 117:35, (1997)), all of which are incorporated herein in their entirety. Corresponding canine antibodies against such lineage antigens have been described by Teske *et al.*, *Exper. Hematol.* 22:1179, (1994). Ladiges *et al.*, *Am. J. Vet. Res.* 49:870, (1988). describes both canine and human antibodies reacting with lineage antigens in canine lymphoma. Both of these documents also are incorporated herein in their entirety.

[0003] Bispecific antibody fusion proteins which bind to these various antigens can be used according to the present invention, including hybrid antibodies which bind to more than one B-cell, T-cell, myeloid-cell, mast-cell or plasma-cell antigen. Preferably the bispecific and hybrid antibodies target either a B-cell antigen, a T-cell, a myeloid-cell, a mast-cell, a plasma-cell or a macrophage antigen.

[0004] In accordance with the teaching of the present invention, B-cell, T-cell, myeloid, mast-cell or plasma-cell disorders can be treated with antibody therapy. The B-cell, T-cell, myeloid, mast cell, or plasma cell disorder can be treated with its respective normal-cell

antibodies directed against the equivalent cell antigens in domestic animals, or an autoimmune disease associated with B-cells or T-cells, particularly a Class III autoimmune disease, can be treated with equivalent B-cell, T-cell or HLA-DR antigens in domestic animals.

[0005] In a preferred embodiment, an antibody component is used that comprises an arm that is specific for a low-molecular weight hapten to which a therapeutic agent is conjugated or fused. In this case, the antibody pretargets B-cells, T-cells, myeloid cells, mast cells or plasma cells, and the low-molecular weight hapten with the attached therapeutic agent is administered after the antibody has bound to the targets. Examples of recognizable haptens include, but are not limited to, chelators, such as DTPA, fluorescein isothiocyanate, vitamin B-12, polymers (such as polymeric proteins or carbohydrates), and other moieties to which specific antibodies can be raised.

[0006] Malignancies that can be treated include B-cell lymphomas, B-cell leukemias, T-cell lymphomas, T-cell leukemias, and myeloid leukemias, mastocytomas, and myelomas, as well as certain premalignant conditions related thereto, such as myelodysplasia syndrome (MDS). The therapeutic compositions described herein are particularly useful for treatment of indolent forms of B-cell lymphomas, aggressive forms of B-cell lymphomas, chronic lymphatic or myeloid leukemias, acute lymphatic or myeloid leukemias, diverse plasma cell dyscrasias (such as multiple myeloma), and mastocytomas.

[0007] Plasma cell tumors consist principally of Waldenstrom's macroglobulinemia (WM) and multiple myeloma (MM). CD20 is expressed more in the former than in the latter, since multiple myeloma is a more differentiated cell type proceeding from B-cell lymphomas to Waldenstrom's and then to multiple myeloma (Treon *et al.*, *Ann. Oncol.* 11 (Suppl 1):107, 2000). Antibodies against a number of human markers, including the immunoglobulin idiotype, can be used as targets for antibodies used in the treatment of plasma cell dyscrasias, including WM and MM, such as CD20, CD38, CD54, CD126, HM1.24, and MUC1 (Treon *et al.*, *Semin. Oncol.* 26 (Suppl 14):97, 1999). A high affinity Mab (AT13/5) against CD38, made as a chimeric and CDR-grafted humanized IgG1, as promising antibodies for the therapy of MM was described by Ellis *et al.* (*J. Immunol.* 155:925, 1995). It has been shown that the humanized anti-HM1.24 antibody effectively kills multiple myeloma cells by human effector cell-mediated cytotoxicity (Ono *et al.*, *Mol. Immunol.* 36:387, 1999; Ozaki *et al.*, *Blood* 93:3922, 1999). Regression of murine leukemias and multiple myeloma has also been

accomplished with CD25 antibodies (interleukin-2 receptor alpha) (Onizuka *et al.*, *Cancer Res*. 59:3128, 1999). Two human plasma-cell reactive antibodies, B-B2 and B-B4, which are suggested as candidates for immunotherapy of multiple myeloma, were reported by Vooijs *et al.*, *Cancer Immunol. Immunother.* 42:319 (1996), when these were tested as immunotoxins with saporin.

[0008] Preferred plasma-cell targets in domestic animals are those equivalent to the human CD20, CD22, CD38, HM1.24, and MUC1. WM has also been shown to express CD22 (Behr *et al.* in *Clin. Cancer Res. Suppl.* 5 (Suppl 10):3304s-2214s (1999) and unpublished results of the inventor). CD20 and CD22 are expressed more in WM, while CD38, HM1.24, and MUC1 are expressed in both WM and MM.

[0009] The most common malignant neoplasms in the dog are mast cell tumors. It has been estimated that this tumor type represents between 7% and 21% of all tumors occurring in dogs, which is much higher in frequency than is found in humans. These tumors frequently spread to local lymph nodes, liver, spleen, and bone marrow (London *et al.*, *Exp. Hematol.* 27:689, 1999).

[0010] The c-kit gene encodes a receptor tyrosine kinase for stem cell factor (SCF; c-kit ligand, KL), both of which play a critical role in the differentiation and growth of hematopoietic stem cells (Teyssier-Le Discorde *et al.*, *Leukemia* 11 (Suppl 3):396, 1997). Stem cell factor receptor (SCFR, c-kit) is normally expressed on hematopoietic and mast cells, where it is believed to play a regulatory role in cellular growth and differentiation (London *et al.*, *J. Comp. Pathol.* 115:399, 1996). It has been found that canine mast cell tumors express SCFR, or KIT (c-kit protein), which is a type III transmembrane receptor kinase. The ligand of the KIT, stem cell factor, is a cytokine that stimulates mast cell growth and differentiation. KIT has been detected in both human and canine mast cell malignancies, as can be shown by immunohistochemical methods, such that KIT has been reported as a reliable marker for canine mast cells and undifferentiated mast cell tumors (London *et al.*, *J. Comp. Pathol.* 115:399, 1996; Reguera *et al.*, *Am. J. Dermatopathol.* 22:49, 2000). Antibodies are already available against KIT, and have been used in the immunohistochemical methods described. These kind of antibodies can serve as immunotherapeutics for controlling the growth of mast cell tumors, while the KIT ligand, stem cell factor, can also be used either to deliver cytotoxic agents to its receptor, or to serve as a hapten or hapten component in a bispecific antibody/pretargeting methodology, in which

one arm is directed against the tumor (*e.g.*, KIT or stem cell factor receptor) and the other arm is made against the ligand or a peptide linked to the ligand (stem cell factor). The latter is for improved delivery of drugs, toxins and isotopes, whereas the naked antibody to KIT can be used by itself or in suitable combinations with other antibodies targeting mast cell tumors or with immunoconjugates consisting of antibodies to SCFR linked to a cytotoxic agent.

[0011] Other potential targets for antibodies against mast cell tumors are thioflavine T (Brunnert and Altman, *J. Vet. Diagn. Invest.* 3:245, 1991), and chymotrypsin-like proteinase (Schechter *et al., Arch. Biochem. Biophys.* 262:232, 1988). Antibodies have been made against these substances, and used to detect them in mast cell tumor tissues. These antibodies can then serve as immunotherapeutics and as the basis for immunoconjugates made for the therapy of mast cell tumors.

[0012] A number of myeloid and lymphatic cancers have been documented in dogs and cats, including both chronic lymphatic or myeloid leukemias and acute lymphatic or myeloid leukemias, e.g., acute lymphoid leukemia, acute myelogenous leukemia, acute myelomonocytic leukemia, monocytic leukemia, lymphocytic leukemia, myelocytic leukemia, and chronic granulocytic leukemia. The term "myelocytic leukemia" is synonymous with the terms granulocytic leukemia, myelogenic leukemia, myelogenous leukemia and myeloid leukemia. The etiology of each of these disorders has been well characterized, in particular the cell type(s) with which the disorder is associated. For example, chronic myelocytic leukemia is characterized by an uncontrolled proliferation of myelopoietic cells in the bone marrow and extramedullary sites in which the malignant myeloblast is able to differentiate and give rise to myelocytes, metamyelocytes, band cells and granulocytes, while acute myelocytic leukemia is characterized by an uncontrolled proliferation of myeloblasts which are unable to differentiate into more mature cell-types. The present methods use naked antibodies or antibody conjugates against these cell types to treat the disorder.

[0013] Recent studies suggest that immunotherapy utilizing naked antibodies can be an effective tool for treating these cancers. For example, the use of naked, humanized, anti-CD33 antibodies has proved effective in treating acute myelocytic leukemia and in reducing the residual disease in patients. See Caron *et al.*, *Clin. Cancer Res.*, 4:1421-1428 (1998); Jurcic *et al.*, *Clin. Cancer Res.*, 6:372-380 (2000). U.S. Provisional Appln. 60/223,698

(Goldenberg *et al.*), which is incorporated herein by reference, describes the use of naked antibodies for treating acute myelocytic leukemia (AML), acute promyelocytic leukemia (APML) and chronic myelocytic leukemia (CML). The method uses naked granulocyte-specific antibodies that recognize an antigen that is present on two or more cell-types of the granulocyte/myelocyte lineage, alone or in conjunction with immunoconjugates or other therapeutics to destroy myeloid leukemia cells. The present invention contemplates the use of myelocyte-specific, metamyelocyte-specific, band-specific antibodies to treat myeloid leukemias, and anti-granulocytic antibodies directed to antigens present on a single granulocyte precursor, such as anti-CD33 or anti-CD15 antibodies, or on two or more cell-types in the granulocyte/myelocyte lineage. The antibodies may be in the form of naked antibodies or immunoconjugates.

[0014] The present invention is also useful in the treatment of autoimmune diseases. B-cell clones that bear autoantibody Ig-receptors are present in normal individuals. Autoimmunity results when these B-cells become overactive, and mature to plasma cells that secrete autoantibody. Other autoimmune disorders are tied to a T-cell disorder. In accordance with the present invention, autoimmune disorders can be treated by administering an antibody that binds to a B-cell or T-cell receptor or antigen, or to an Ia or HLA-DR determinant or equivalent in domestic animals. In one embodiment, comparatively low doses of an entire, naked antibody or combinations of entire, naked antibodies are used. In other embodiments, immunoconjugates or fusion proteins of antibody components with drugs, toxins or therapeutic radioisotopes are useful.

[0015] The antibodies described in this invention can be antibodies from the same species as the animal to be treated. Preferred monoclonal antibodies can be made by immunizing an animal of the species to be treated, and fusing the resulting antibodies with a myeloma cell line, either from the same species or from another species, such as a mouse. In a preferred embodiment, molecular engineering methods can be used to graft mouse or other species' CDRs to dog or cat (or other domestic species) framework regions. Humanized antibodies can also be used in accordance with the present invention. Alternatively, mouse, mouse/human chimeric, and fully human monoclonal antibodies, as well as dog, mouse/dog chimeric, or caninized monoclonal antibodies, as described above, can also be used. Fully canine, feline, equine or other domestic species antibodies, as may be preferred for a particular animal species to be treated, may be made by methods similar to those employed to

construct or isolate fully human antibodies, such as by phage display and gene or chromosome transfer techniques.

[0016] The antibodies can be infused into a domestic animal diagnosed with a B-cell, T-cell, myeloid-, plasma-, or mast-cell disorder, in order to treat or control the disorder. Where the disorder is a malignancy, the malignancy will be decreased in size as a result of the treatment, and the animal may even enter a state of total remission. Where the disorder is an autoimmune disease, treatment is generally an ongoing treatment of a chronic condition.

[0017] In a preferred embodiment, naked antibodies are used alone in treatment. Combinations of naked antibodies may be used, *i.e.*, naked antibodies to more than one epitope of a B-cell antigen or a T-cell antigen, or even combinations against distinctly different B-cell or T-cell antigens, may be used. In some cases, it may be preferable to use a naked antibody that binds to both B-cell and T-cell antigens. For example, certain HLA antibodies may bind to both. Other examples include human-equivalent lymphocyte antibodies, such as against CD21 in the dog.

[0018] In another embodiment, naked antibody therapy can be enhanced by supplementing the naked antibodies with immunoconjugates comprising an antibody component and a therapeutic agent, or with fusion proteins, conventional chemotherapeutic drugs, cytokines, and other forms of supplemental therapy. In such multimodal regimens, the naked antibodies are the primary therapeutic, and the supplemental therapeutic compositions can be administered before, concurrently or after administration of one or more naked antibodies.

[0019] Immunoconjugates also may be used alone for treatment. In one embodiment, the immunoconjugate is a radiolabeled immunoconjugate. The radionuclide may be an alpha emitter, a beta-emitter, and/or an Auger emitter. Suitable radionuclides include ^{198}Au, ^{32}P, ^{125}I, ^{131}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{67}Cu, ^{177}Lu, ^{211}At, ^{213}Bi, ^{111}In, ^{67}Ga, and ^{225}Ac. In another embodiment, the immunoconjugate comprises a drug or toxin. Suitable drugs or toxins include ricin, abrin, ribonuclease, DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin. The immunoconjugate therapy may be supplemented with conventional chemotherapy, radiation therapy, and/or cytokine therapy.

[0020] In a further embodiment, imaging is performed prior to therapy to confirm the presence and/or location of the disease. Examples of diagnostic agents include, but are not

limited to, radioisotopes, coloring agents (such as the biotin-streptavidin complex), contrasting agents, fluorescent compounds or molecules and enhancing agents for magnetic resonance imaging (MRI). Preferably, the diagnostic agents are selected from the group consisting of radioisotopes, enhancing agents for use in magnetic resonance imaging, and fluorescent compunds.

[0021] In order to load an antibody component with radioactive metals or paramagnetic ions, it may be necessary to react it with a reagent having a long tail to which are attached a multiplicity of chelating groups for binding the ions. Such a tail can be a polymer such as a polylysine, polysaccharide, or other derivatized or derivatizable chain having pendant groups to which can be bound chelating groups such as, *e.g.,* ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), porphyrins, polyamines, crown ethers, bis-thiosemicarbazones, polyoximes, and like groups known to be useful for this purpose. Chelates are coupled to the peptide antigens using standard chemistries. The chelate is normally linked to the antibody by a group which enables formation of a bond to the molecule with minimal loss of immunoreactivity and minimal aggregation and/or internal cross-linking. other, more unusual, methods and reagents for conjugating chelates to antibodies are disclosed in U.S. Patent 4,824,659 to Hawthorne, entitled "Antibody Conjugates", issued April 25, 1989, the disclosure of which is incorporated herein in its entirety by reference.

[0022] Particularly useful metal-chelate combinations include 2-benzyl-DTPA and its monomethyl and cyclohexyl analogs, used with scandium-47, iron-52, cobalt-55, gallium-67, gallium-68, indium-111, zirconium-89, yttrium-90, terbium-161, lutetium-177, bismuth-212, bismuth-213, and actinium-225 for radio-imaging and RAIT. The same chelates, when complexed with non-radioactive metals, such as manganese, iron and gadolinium are useful for MRI, when used along with the antibodies of the invention. Macrocyclic chelates such as NOTA, DOTA, and TETA are of use with a variety of metals and radiometals, most particularly with radionuclides of gallium, ytrrium and copper, respectively. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Other ring-type chelates such as macrocyclic polyethers, which are of interest for stably binding nuclides such as radium-223 for RAIT are encompassed by the invention.

[0023] As in the case of therapeutic antibodies, an antibody component can be used that comprises an arm that is specific for a low-molecular weight hapten to which the diagnostic

agent is conjugated or fused. In this case, the antibody component pretargets B-cells, T-cells, myeloid cells, mast cells or plasma cells, and the low-molecular weight hapten with the attached diagnostic agent is administered after the antibody has bound to the target.

[0024] MRI contrast agents are well known in the art and include, for example, Gadolinium, Iron, Manganese, Rhenium, Europium, Lanthanium, Holmium, and Ferbium. Where a radionuclide is use, the antibody component comprises a gamma-emitting diagnostic radionuclide. The diagnostic radionuclide is selected from the group consisting of ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{99}Tc, ^{197}Hg, ^{67}Ga, ^{68}Ga, ^{191}Os, ^{111}In, ^{113}In and ^{203}Pb. Some therapeutic radionuclides have a companion gamma energy component which can be used for diagnostic imaging. In this case, imaging occurs in conjunction with therapy.

[0025] **Definitions**

[0026] In the description that follows, and in the documents incorporated by reference herein, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

[0027] A **structural gene** is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

[0028] A **promoter** is a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5′ region of a gene, proximal to the transcriptional start site of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

[0029] An **isolated DNA molecule** is a fragment of DNA that is not integrated in the genomic DNA of an organism. For example, a cloned antibody gene is a DNA fragment that has been separated from the genomic DNA of a mammalian cell. Another example of an isolated DNA molecule is a chemically-synthesized DNA molecule that is not integrated in the genomic DNA of an organism.

[0030] An **enhancer** is a DNA regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

[0031] **Complementary DNA** (cDNA) is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand.

[0032] The term **expression** refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

[0033] A **cloning vector** is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

[0034] An **expression vector** is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

[0035] A **recombinant host** may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

[0036] An **antibody fragment** is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab′, Fab, and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, an anti-CD22 monoclonal antibody fragment binds with an epitope of CD22.

[0037] The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For

example, antibody fragments include isolated fragments consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("sFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

[0038] A **chimeric antibody** is a recombinant protein that contains the variable domains and complementary determining regions derived from one species while the remainder of the antibody molecule is derived from another species antibody.

[0039] **Humanized antibodies** are recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

[0040] **Caninized or felinized antibodies** are recombinant proteins in which rodent (or another species) complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of rodent (or another species) immunoglobulin into a dog or cat, respectively, variable domain.

[0041] As used herein, a **therapeutic agent** is a molecule or atom that is conjugated to an antibody moiety to produce a conjugate that is useful for therapy. Examples of therapeutic agents include drugs, toxins, antagonists, enzymes, immunomodulators, hormones, cytokines, chelators, boron compounds, photoactive agents or dyes, and radioisotopes.

[0042] A **naked antibody** is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized, caninized, or felinized antibodies, as well as fully human, canine, or feline antibodies derived from recombinant engineering or other genetic or chromosomal manipulation technologies.

[0043] As used herein, the term **antibody component** includes both an entire antibody and an antibody fragment.

[0044] An **immunoconjugate** is a conjugate of an antibody component with a therapeutic agent.

[0045] A **fusion protein** is a recombinantly produced antigen-binding molecule in which two or more different single-chain antibody or antibody fragment segments with the same or different specificities are linked. A variety of bispecific fusion proteins can be produced using molecular engineering. In one form, the bispecific fusion protein is monovalent, consisting of, for example, a scFv with a single binding site for one antigen and a Fab fragment with a single binding site for a second antigen. In another form, the bispecific fusion protein is divalent, consisting of, for example, an IgG with two binding sites for one antigen and two scFv with two binding sites for a second antigen. The fusion protein additionally comprises a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators ("antibody-immunomodulator fusion protein") and toxins ("antibody-toxin fusion protein"). One preferred toxin comprises a ribonuclease (RNase), preferably a recombinant RNase. The fusion protein may comprise a single antibody component, a multivalent combination of different antibody components or multiple copies of the same antibody component.

[0046] A **multispecific antibody** is an antibody that can bind simultaneously to at least two targets that are of different structure, *e.g.*, two different antigens, two different epitopes on the same antigen, or a hapten and/or an antigen or epitope. One specificity would be for a B-cell, T-cell, myeloid-, plasma-, and mast-cell antigen or epitope. Another specificity could be to a different antigen on the same cell type, such as CD19 and CD20 on B-cells.

[0047] A **bispecific antibody** is an antibody that can bind simultaneously to two targets which are of different structure. Bispecific antibodies (bsAb) and bispecific antibody fragments (bsFab) have at least one arm that specifically binds to, for example, a B-cell, T-cell, myeloid-, plasma-, and mast-cell antigen or epitope and at least one other arm that specifically binds to a targetable conjugate that bears a therapeutic or diagnostic agent.

[0048] **Domestic animals** include large animals such as horses, cattle, sheep, goats, llamas, alpacas, and pigs, as well as companion animals. In a preferred embodiment, the domestic animal is a horse.

[0049] **Companion animals** include animals kept as pets. These are primarily dogs and cats, although small rodents, such as guinea pigs, hamsters, rats, and ferrets, are also included, as are subhuman primates such as monkeys. In a preferred embodiment the companion animal is a dog or a cat.

[0050] Production of Naked Antibodies

[0051] Naked antibodies to any known antigen that is characteristics of a B-cell or T-cell disorder can be used in accordance with the present invention. Many monoclonal antibodies to B-cell and T-cell antigens are available, such as 1F5, L243, Leu-16 and ACT1. A clone to make 1F5 anti-CD20 mAb can be obtained from ATCC (HB-96450). A clone to make L243, an MHC class II antibody, can be obtained from ATCC (HB-55). Leu-16, an anti-CD3 antibody, can be obtained from Becton-Dickinson. Clones to make anti-IL-2 receptor mAbs include 2A3A1H from ATCC (HB8555) and 7G7B6 from ATCC (HB8784). The ACT1 antibody is described in Galkowska *et al.*, *Vet. Immunol. Immunopathol.*, 53;329-324 (1996), which further reports that many anti-human antibodies are species cross-reactive. Thus, anti-human antibodies can be used in methods according to the invention. See, also, *Shienvold et al.*, ("Antibodies against Ia-like antigens have shown cross-species properties" *Transplantation* 41:364 (1986)) which shows that monoclonal antibodies directed against Ia show crossreactivity among different species. For example, the B1F6 antibody shows reactivity with Ia in the dog, rat, pig and human, and B2E8 shows reactivity with Ia in the dog, pig and human.

[0052] Rodent monoclonal antibodies to CD22 or CD19 can be obtained by methods known to those skilled in the art. See generally, for example, Kohler and Milstein, *Nature 256*:495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"]. The production of CD22 and CD19 according to the following protocol is exemplary of techniques for the production of antibodies to any lineage or non-lineage human antigen or its cross-species equivalent. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising CD22 or CD19, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce anti-CD22 or anti-CD19 antibodies, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

[0053] Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-

2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

[0054] Suitable amounts of the well-characterized CD22 or CD19 antigen for production of antibodies can be obtained using standard techniques. As an example, CD22 can be immunoprecipitated from B-lymphocyte protein using the deposited antibodies described by Tedder *et al.*, U.S. patent No. 5,484,892 (1996).

[0055] Alternatively, CD22 protein or CD19 protein can be obtained from transfected cultured cells that overproduce CD22 or CD19. Expression vectors that comprise DNA molecules encoding CD22 or CD19 proteins can be constructed using published CD22 and CD19 nucleotide sequences. See, for example, Wilson *et al., J. Exp. Med. 173*:137 (1991); Wilson *et al., J. Immunol. 150*:5013 (1993). As an illustration, DNA molecules encoding CD22 or CD19 can be obtained by synthesizing DNA molecules using mutually priming long oligonucleotides. See, for example, Ausubel *et al.,* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, pages 8.2.8 to 8.2.13 (1990) ["Ausubel"]. Also, see Wosnick *et al., Gene 60*:115 (1987); and Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 8-8 to 8-9 (John Wiley & Sons, Inc. 1995). Established techniques using the polymerase chain reaction provide the ability to synthesize genes as large as 1.8 kilobases in length. Adang *et al., Plant Molec. Biol. 21*:1131 (1993); Bambot *et al., PCR Methods and Applications 2*:266 (1993); Dillon *et al.,* "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 263-268, (Humana Press, Inc. 1993).

[0056] In a variation of this approach, anti-CD22 or anti-CD19 monoclonal antibody can be obtained by fusing myeloma cells with spleen cells from mice immunized with a murine pre-B cell line stably transfected with CD22 cDNA or CD19 cDNA. See Tedder *et al.*, U.S. patent No. 5,484,892 (1996).

[0057] One example of a suitable murine anti-CD22 monoclonal antibody is the LL2 (formerly EPB-2) monoclonal antibody, which was produced against human Raji cells derived from a Burkitt lymphoma. Pawlak-Byczkowska *et al., Cancer Res. 49*:4568 (1989). This monoclonal antibody has an $IgG_{2\alpha}$ isotype, and the antibody is rapidly internalized into lymphoma cells. Shih *et al, Int. J. Cancer 56*:538 (1994). Immunostaining and *in vivo*

radioimmunodetection studies have demonstrated the excellent sensitivity of LL2 in detecting B-cell lymphomas. Pawlak-Byczkowska *et al., Cancer Res. 49*:4568 (1989); Murthy *et al., Eur. J. Nucl. Med. 19*:394 (1992). Moreover, ^{99m}Tc-labeled LL2-Fab' fragments have been shown to be useful in following upstaging of B-cell lymphomas, while ^{131}I-labeled intact LL2 and labeled LL2 $F(ab')_2$ fragments have been used to target lymphoma sites and to induce therapeutic responses. Murthy *et al., Eur. J. Nucl. Med. 19*:394 (1992); Mills *et al., Proc. Am. Assoc. Cancer Res. 34*:479 (1993) [Abstract 2857]; Baum *et al., Cancer 73 (Suppl. 3)*:896 (1994); Goldenberg *et al., J. Clin. Oncol. 9*:548 (1991). Furthermore, Fab' LL2 fragments conjugated with a derivative of *Pseudomonas* exotoxin has been shown to induce complete remissions for measurable human lymphoma xenografts growing in nude mice. Kreitman *et al., Cancer Res. 53*:819 (1993).

[0058] In an additional embodiment, an antibody of the present invention is a chimeric antibody in which the variable regions of a target species antibody have been replaced by the variable regions of a rodent anti-CD20 or anti-CD19 antibody. The advantages of chimeric antibodies include decreased immunogenicity and increased *in vivo* stability.

[0059] Techniques for constructing chimeric antibodies are well known to those of skill in the art. As an example, Leung *et al., Hybridoma 13:*469 (1994), describe how they produced an LL2 chimera by combining DNA sequences encoding the V_κ and V_H domains of LL2 monoclonal antibody with respective human κ and IgG_1 constant region domains. This publication also provides the nucleotide sequences of the LL2 light and heavy chain variable regions, V_κ and V_H, respectively.

[0060] In yet another embodiment, an antibody of the present invention comprises a monoclonal antibody in which mouse complementarity determining regions are transferred from heavy and light variable chains of the mouse immunoglobulin into the variable domain of the target species, followed by the replacement of some residues of the target species in the framework regions of their murine counterparts. These antibodies are particularly suitable for use in therapeutic methods in the target species. General techniques for cloning murine immunoglobulin variable domains are described, for example, by the publication of Orlandi *et al., Proc. Nat'l Acad. Sci. USA 86*: 3833 (1989). Techniques for producing monoclonal antibodies that are humanized, *i.e.*, where a human is the target species, are described, for example, by Jones *et al., Nature 321*:522 (1986), Riechmann *et al., Nature 332*:323 (1988), Verhoeyen *et al., Science 239*:1534 (1988), Carter *et al., Proc. Nat'l Acad. Sci. USA 89*:4285

(1992), Sandhu, *Crit. Rev. Biotech. 12*:437 (1992), and Singer *et al., J. Immun. 150*:2844 (1993). The publication of Leung *et al., Mol. Immunol. 32*:1413 (1995), describes the construction of humanized LL2 antibody. These techniques can be extended to the construction of antibodies with the variable domain of the horse, dog, cat, or other targeted species.

[0061] In another embodiment, an antibody of the present invention is a target species monoclonal antibody, obtained from transgenic mice that have been "engineered" to produce specific target species antibodies in response to antigenic challenge. In this technique, elements of the target species heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize target species antibodies specific for target species antigens, and the mice can be used to produce target species antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al., Nature Genet. 7*:13 (1994), Lonberg *et al., Nature 368*:856 (1994), and Taylor *et al., Int. Immun. 6*:579 (1994). These techniques can be extended to the engineering of antibodies with the variable domain of the horse, dog, cat, or other targeted species.

[0062] In another embodiment, an antibody of the present invention is raised in another individual of the same species as the species that is the target of the therapy. General techniques for raising therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465 (1991), and in Losman *et al., Int. J. Cancer 46*: 310 (1990), and these techniques can be extended to the production of antibodies in other species.

[0063] **Production of Antibody Fragments**

[0064] The present invention contemplates the use of fragments of antibodies or other therapeutically useful antibody components. Antibody fragments can be prepared by proteolytic hydrolysis of an antibody or by expression in *E. coli* of the DNA coding for the fragment.

[0065] Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This

fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab′ monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff *et al., Arch Biochem. Biophys. 89*:230 (1960); Porter, *Biochem. J. 73*:119 (1959), Edelman *et al.,* in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

[0066] Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

[0067] For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described in Inbar *et al., Proc. Nat'l Acad. Sci. USA 69*:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, for example, Sandhu, *supra*.

[0068] Preferably, the Fv fragments comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al., Methods: A Companion to Methods in Enzymology 2*:97 (1991). See also Bird *et al., Science 242*:423 (1988), Ladner *et al.,* U.S. Patent No. 4,946,778, Pack *et al., Bio/Technology 11*:1271 (1993), and Sandhu, *supra*.

[0069] Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the

variable region from RNA of antibody-producing cells. See, for example, Larrick *et al., Methods: A Companion to Methods in Enzymology 2*:106 (1991); Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 166-179 (Cambridge University Press 1995); and Ward *et al.,* "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.*, (eds.), pages 137-185 (Wiley-Liss, Inc. 1995).

[0070] **Preparation of Immunoconjugates**

[0071] The present invention contemplates the use of "naked" antibodies, as well as the use of immunoconjugates to effect treatment of B-cell, T-cell, myeloid-, plasma-, and mast-cell disorders. Such immunoconjugates can be prepared by indirectly conjugating a therapeutic agent to an antibody component. General techniques are described in Shih *et al., Int. J. Cancer 41*:832-839 (1988); Shih *et al., Int. J. Cancer 46*:1101-1106 (1990); and Shih *et al.*, U.S. patent No. 5,057,313. The general method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function and that is loaded with a plurality of drug, toxin, chelator, boron addends, or other therapeutic agent. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

[0072] The carrier polymer is preferably an aminodextran or polypeptide of at least 50 amino acid residues, although other substantially equivalent polymer carriers can also be used. Preferably, the final immunoconjugate is soluble in an aqueous solution, such as mammalian serum, for ease of administration and effective targeting for use in therapy. Thus, solubilizing functions on the carrier polymer will enhance the serum solubility of the final immunoconjugate. In particular, an aminodextran will be preferred.

[0073] The process for preparing an immunoconjugate with an aminodextran carrier typically begins with a dextran polymer, advantageously a dextran of average molecular weight of about 10,000 - 100,000. The dextran is reacted with an oxidizing agent to effect a controlled oxidation of a portion of its carbohydrate rings to generate aldehyde groups. The oxidation is conveniently effected with glycolytic chemical reagents such as $NaIO_4$, according to conventional procedures.

[0074] The oxidized dextran is then reacted with a polyamine, preferably a diamine, and more preferably, a mono- or polyhydroxy diamine. Suitable amines include ethylene diamine, propylene diamine, or other like polymethylene diamines, diethylene triamine or like polyamines, 1,3-diamino-2-hydroxypropane, or other like hydroxylated diamines or polyamines, and the like. An excess of the amine relative to the aldehyde groups of the dextran is used to insure substantially complete conversion of the aldehyde functions to Schiff base groups.

[0075] A reducing agent, such as $NaBH_4$, $NaBH_3CN$ or the like, is used to effect reductive stabilization of the resultant Schiff base intermediate. The resultant adduct can be purified by passage through a conventional sizing column to remove cross-linked dextrans.

[0076] Other conventional methods of derivatizing a dextran to introduce amine functions can also be used, *e.g.*, reaction with cyanogen bromide, followed by reaction with a diamine.

[0077] The aminodextran is then reacted with a derivative of the particular drug, toxin, chelator, immunomodulator, boron addend, or other therapeutic agent to be loaded, in an activated form, preferably, a carboxyl-activated derivative, prepared by conventional means, *e.g.*, using dicyclohexylcarbodiimide (DCC) or a water soluble variant thereof, to form an intermediate adduct.

[0078] Alternatively, polypeptide toxins such as pokeweed antiviral protein or ricin A-chain, and the like, can be coupled to aminodextran by glutaraldehyde condensation or by reaction of activated carboxyl groups on the protein with amines on the aminodextran.

[0079] Chelators for radiometals or magnetic resonance enhancers are well-known in the art. Typical are derivatives of ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). These chelators typically have groups on the side chain by which the chelator can be attached to a carrier. Such groups include, *e.g.*, benzylisothiocyanate, by which the DTPA or EDTA can be coupled to the amine group of a carrier. Alternatively, carboxyl groups or amine groups on a chelator can be coupled to a carrier by activation or prior derivatization and then coupling, all by well-known means.

[0080] Boron addends, such as carboranes, can be attached to antibody components by conventional methods. For example, carboranes can be prepared with carboxyl functions on pendant side chains, as is well known in the art. Attachment of such carboranes to a carrier,

e.g., aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and condensation with amines on the carrier to produce an intermediate conjugate. Such intermediate conjugates are then attached to antibody components to produce therapeutically useful immunoconjugates, as described below.

[0081] A polypeptide carrier can be used instead of aminodextran, but the polypeptide carrier must have at least 50 amino acid residues in the chain, preferably 100-5000 amino acid residues. At least some of the amino acids should be lysine residues or glutamate or aspartate residues. The pendant amines of lysine residues and pendant carboxylates of glutamine and aspartate are convenient for attaching a drug, toxin, immunomodulator, chelator, boron addend or other therapeutic agent. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, *e.g.*, serines, to confer desirable solubility properties on the resultant loaded carrier and immunoconjugate.

[0082] Conjugation of the intermediate conjugate with the antibody component is effected by oxidizing the carbohydrate portion of the antibody component and reacting the resulting aldehyde (and ketone) carbonyls with amine groups remaining on the carrier after loading with a drug, toxin, chelator, immunomodulator, boron addend, or other therapeutic agent. Alternatively, an intermediate conjugate can be attached to an oxidized antibody component via amine groups that have been introduced in the intermediate conjugate after loading with the therapeutic agent. Oxidation is conveniently effected either chemically, *e.g.*, with $NaIO_4$ or other glycolytic reagent, or enzymatically, *e.g.*, with neuraminidase and galactose oxidase. In the case of an aminodextran carrier, not all of the amines of the aminodextran are typically used for loading a therapeutic agent. The remaining amines of aminodextran condense with the oxidized antibody component to form Schiff base adducts, which are then reductively stabilized, normally with a borohydride reducing agent.

[0083] Analogous procedures are used to produce other immunoconjugates according to the invention, as well as other procedures known in the art. Loaded polypeptide carriers preferably have free lysine residues remaining for condensation with the oxidized carbohydrate portion of an antibody component. Carboxyls on the polypeptide carrier can, if necessary, be converted to amines by, *e.g.*, activation with DCC and reaction with an excess of a diamine.

[0084] The final immunoconjugate is purified using conventional techniques, such as sizing chromatography on Sephacryl S-300.

[0085] Alternatively, immunoconjugates can be prepared by directly conjugating an antibody component with a therapeutic agent. The general procedure is analogous to the indirect method of conjugation except that a therapeutic agent is directly attached to an oxidized antibody component.

[0086] It will be appreciated that other therapeutic agents can be substituted for the chelators described herein. Those of skill in the art will be able to devise conjugation schemes without undue experimentation.

[0087] As a further illustration, a therapeutic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation. For example, the tetanus toxoid peptides can be constructed with a single cysteine residue that is used to attach the peptide to an antibody component. As an alternative, such peptides can be attached to the antibody component using a heterobifunctional cross-linker, such as *N*-succinyl 3-(2-pyridyldithio)proprionate (SPDP). Yu *et al., Int. J. Cancer 56*:244 (1994). General techniques for such conjugation are well known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991); Upeslacis *et al.*, "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.* (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 60-84 (Cambridge University Press 1995).

[0088] As described above, carbohydrate moieties in the Fc region of an antibody can be used to conjugate a therapeutic agent. However, the Fc region is absent if an antibody fragment is used as the antibody component of the immunoconjugate. Nevertheless, it is possible to introduce a carbohydrate moiety into the light chain variable region of an antibody or antibody fragment. See, for example, Leung *et al., J. Immunol. 154*:5919 (1995); Hansen *et al.*, U.S. patent No. 5,443,953 (1995). The engineered carbohydrate moiety is then used to attach a therapeutic agent.

[0089] In addition, those of skill in the art will recognize numerous possible variations of the conjugation methods. For example, the carbohydrate moiety can be used to attach polyethyleneglycol in order to extend the half-life of an intact antibody, or antigen-binding fragment thereof, in blood, lymph, or other extracellular fluids. Moreover, it is possible to construct a "divalent immunoconjugate" by attaching therapeutic agents to a carbohydrate moiety and to a free sulfhydryl group. Such a free sulfhydryl group may be located in the hinge region of the antibody component.

[0090] Preparation of Fusion Proteins

[0091] The present invention further contemplates the use of fusion proteins comprising one or more antibody moieties. In addition to antibody components that bind with a first B-cell, T-cell, myeloid, mast-cell, or plasma-cell antigen or epitope, useful antibody moieties may include antibody components that bind with other B-cell, T-cell, myeloid, mast cell or plasma cell antigens or epitopes, and a fusion protein may comprise one, two, three, four or even more antibody types. Bivalent, trivalent, tetravalent and quatravalent constructs can be used in accordance with the invention. These fusion proteins optionally comprise an immunomodulator or toxin moiety.

[0092] Methods of making antibody-immunomodulator fusion proteins are known to those of skill in the art. For example, antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti *et al., Ann. Oncol. 6*:945 (1995), Nicolet *et al., Cancer Gene Ther. 2*:161 (1995), Becker *et al., Proc. Nat'l Acad. Sci. USA 93*:7826 (1996), Hank *et al., Clin. Cancer Res. 2*:1951 (1996), and Hu *et al., Cancer Res. 56*:4998 (1996). In addition, Yang *et al., Hum. Antibodies Hybridomas 6*:129 (1995), describe a fusion protein that includes an $F(ab')_2$ fragment and a tumor necrosis factor-alpha moiety.

[0093] Methods of making antibody-toxin fusion proteins in which a recombinant molecule comprises one or more antibody components and a toxin or chemotherapeutic agent also are known to those of skill in the art. For example, antibody-*Pseudomonas* exotoxin A fusion proteins have been described by Chaudhary *et al., Nature 339*:394 (1989), Brinkmann *et al., Proc. Nat'l Acad. Sci. USA 88*:8616 (1991), Batra *et al., Proc. Nat'l Acad. Sci. USA 89*:5867 (1992), Friedman *et al., J. Immunol. 150*:3054 (1993), Wels *et al., Int. J. Can. 60*:137 (1995), Fominaya *et al., J. Biol. Chem. 271*:10560 (1996), Kuan *et al., Biochemistry 35*:2872 (1996), and Schmidt *et al., Int. J. Can. 65*:538 (1996). Antibody-toxin fusion proteins containing a diphtheria toxin moiety have been described by Kreitman *et al., Leukemia 7*:553 (1993),

Nicholls *et al., J. Biol. Chem. 268*:5302 (1993), Thompson *et al., J. Biol. Chem. 270*:28037 (1995), and Vallera *et al., Blood 88*:2342 (1996). Deonarain *et al., Tumor Targeting 1*:177 (1995), have described an antibody-toxin fusion protein having an RNase moiety, while Linardou *et al., Cell Biophys. 24-25*:243 (1994), produced an antibody-toxin fusion protein comprising a DNase I component. Gelonin was used as the toxin moiety in the antibody-toxin fusion protein of Wang *et al.*, Abstracts of the 209th ACS National Meeting, Anaheim, CA, 2-6 April, 1995, Part 1, BIOT005. As a further example, Dohlsten *et al., Proc. Nat'l Acad. Sci. USA 91*:8945 (1994), reported an antibody-toxin fusion protein comprising *Staphylococcal* enterotoxin-A.

[0094] Illustrative of toxins which are suitably employed in the preparation of such conjugates are ricin, abrin, ribonuclease RNase), DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin. See, for example, Pastan *et al., Cell 47*:641 (1986), and Goldenberg, *CA - A Cancer Journal for Clinicians 44*:43 (1994). Other suitable toxins and conjugation methods are known to those of skill in the art.

[0095] **Coupling of Antibodies, Immunoconjugates and Fusion Proteins to Lipid Emulsions**

[0096] Long-circulating sub-micron lipid emulsions, stabilized with poly(ethylene glycol)-modified phosphatidylethanolamine (PEG-PE), can be used as drug carriers for the naked antibodies, immunoconjugates, and fusion proteins of the present invention. The emulsions are composed of two major parts: an oil core, *e.g.*, triglyceride, stabilized by emulsifiers, *e.g.*, phospholipids. The poor emulsifying properties of phospholipids can be enhanced by adding a biocompatible co-emulsifier such as polysorbate 80. In a preferred embodiment, the naked antibodies, immunoconjugates and/or fusion proteins are conjugated to the surface of the lipid emulsion globules with a poly(ethylene glycol)-based, heterobifunctional coupling agent, poly(ethylene glycol)-vinylsulfone-N-hydroxy-succinimidyl ester (NHS-PEG-VS).

[0097] The submicron lipid emulsion is prepared and characterized as described. Lundberg, *J. Pharm. Sci.*, **83**:72 (1993); Lundberg *et al., Int. J. Pharm.*, **134**:119 (1996). The basic composition of the lipid emulsion is triolein:DPPC:polysorbate 80, 2:1:0.4 (w/w). When indicated, PEG-DPPE is added into the lipid mixture at an amount of 2-8 mol% calculated on DPPC.

[0098] The coupling procedure starts with the reaction of the NHS ester group of NHS-PEG-VS with the amino group of distearoyl phosphatidyl-ethanolamine (DSPE). Twenty-five μmol of NHS-PEG-VS are reacted with 23 μmol of DSPE and 50 μmol triethylamine in 1 ml of chloroform for 6 hours at 40°C to produce a poly(ethylene glycol) derivative of phosphatidyl-ethanolamine with a vinylsulfone group at the distal terminus of the poly(ethylene glycol) chain (DSPE-PEG-VS). For antibody conjugation, DSPE-PEG-VS is included in the lipid emulsion at 2 mol% of DPPC. The components are dispersed into vials from stock solutions at -20°C, the solvent is evaporated to dryness under reduced pressure. Phosphate-buffered saline (PBS) is added, the mixture is heated to 50°C, vortexed for 30 seconds and sonicated with a MSE probe sonicator for 1 minute. Emulsions can be stored at 4°C, and preferably are used for conjugation within 24 hours.

[0099] Coupling of antibodies to emulsion globules is performed via a reaction between the vinylsulfone group at the distal PEG terminus on the surface of the globules and free thiol groups on the antibody. Vinylsulfone is an attractive derivative for selective coupling to thiol groups. At approximately neutral pH, VS will couple with a half life of 15-20 minutes to proteins containing thiol groups. The reactivity of VS is slightly less than that of maleimide, but the VS group is more stable in water and a stable linkage is produced from reaction with thiol groups.

[00100] Before conjugation, the antibody is reduced by 50 mM 2-mercaptoethanol for 10 minutes at 4°C in 0.2 M Tris buffer (pH 8.7). The reduced antibody is separated from excess 2-mercaptoethanol with a Sephadex G-25 spin column, equilibrated in 50 mM sodium acetate buffered 0.9% saline (pH 5.3). The product is assayed for protein concentration by measuring its absorbance at 280 nm (and assuming that a 1 mg/ml antibody solution of 1.4) or by quantitation of ^{125}I-labeled antibody. Thiol groups are determined with Aldrithiol™ following the change in absorbance at 343 nm and with cystein as standard.

[00101] The coupling reaction is performed in HEPES-buffered saline (pH 7.4) overnight at ambient temperature under argon. Excess vinylsulfone groups are quenched with 2 mM 2-mercaptoethanol for 30 minutes, excess 2-mercaptoethanol and antibody are removed by gel chromatography on a Sepharose CL-48 column. The immunoconjugates are collected near the void volume of the column, sterilized by passage through a 0.45 μm sterile filter, and stored at 4°C.

[00102] Coupling efficiency is calculated using ^{125}I-labeled antibody. Recovery of emulsions is estimated from measurements of [^{14}C]DPPC in parallel experiments. The conjugation of reduced LL2 to the VS group of surface-grafted DSPE-PEG-VS is very reproducible with a typical efficiency of near 85%.

[00103] **Therapeutic Use of Antibodies in Simple and Multimodal Regimens**

[00104] The present invention contemplates the use of naked antibodies, or immunoconjugates or fusion proteins, as the primary therapeutic composition for treatment of B-cell, T-cell, myeloid, mast-cell, and plasma-cell disorders. Such a composition can contain polyclonal antibodies or monoclonal antibodies.

[00105] A therapeutic composition of the present invention can contain a monoclonal antibody directed to non-blocking B-cell, T-cell, myeloid-, plasma-, and mast-cell antigens or epitopes, or it can contain a mixture of monoclonal antibodies directed to different, non-blocking B-cell or T-cell antigens or epitopes. For example, monoclonal antibody cross-inhibition studies have identified five epitopes on CD22, designated as epitopes A-E. See, for example, Schwartz-Albiez *et al.*, "The Carbohydrate Moiety of the CD22 Antigen Can Be Modulated by Inhibitors of the Glycosylation Pathway," in LEUKOCYTE TYPING IV. WHITE CELL DIFFERENTIATION ANTIGENS, Knapp *et al.* (eds.), p. 65 (Oxford University Press 1989). As an illustration, the LL2 antibody binds with epitope B. Stein *et al., Cancer Immunol. Immunother. 37*:293 (1993). Accordingly, therapeutic compositions comprising a mixture of monoclonal anti-CD22 antibodies that bind at least two CD22 epitopes could be used. For example, such a mixture can contain monoclonal antibodies that bind with at least two CD22 epitopes selected from the group consisting of epitope A, epitope B, epitope C, epitope D and epitope E. Similarly, the present invention contemplates therapeutic compositions comprising a mixture of monoclonal anti-CD19 antibodies that bind at least two CD19 epitopes, and so forth.

[00106] Methods for determining the binding specificity of a particular antibody are well known to those of skill in the art. General methods for anti-CD22 antibodies are provided, for example, by Mole, "Epitope Mapping," in METHODS IN MOLECULAR BIOLOGY, VOLUME 10: IMMUNOCHEMICAL PROTOCOLS, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992). More specifically, competitive blocking assays to determine CD22 epitope specificity are described by Stein *et al., Cancer Immunol. Immunother. 37*:293 (1993), and by Tedder *et al.*, U.S. patent No. 5,484,892 (1996).

[00107] The Tedder patent also describes the production of CD22 mutants which lack one or more immunoglobulin-like domains. These mutant proteins were used to determine that immunoglobulin-like domains 1, 2, 3, and 4 correspond with epitopes A, D, B, and C, respectively. Thus, CD22 epitope specificity can also be identified by binding a test antibody with a panel of CD22 proteins lacking particular immunoglobulin-like domain. These techniques may be extended to other antibodies.

[00108] Naked antibodies can be used as the primary therapeutic compositions for treatment of B-cell, T-cell, myeloid, mast cell, or plasma cell malignancies, although the efficacy of such antibody therapy can be enhanced by supplementing naked antibodies with immunoconjugates, fusion proteins, and other forms of supplemental therapy described herein. Multimultimodal regimens with naked antibodies, the supplemental therapeutic compositions can be administered before, concurrently or after administration of the naked antibodies. Alternatively, the B-cell or T-cell, myeloid, mast cell or plasma cell disorder can be treated with immunoconjugates or fusion proteins, without the use of naked antibodies.

[00109] The therapeutic compositions described herein are particularly useful for treatment of indolent forms of B-cell lymphomas, aggressive forms of B-cell lymphomas, chronic and acute lymphatic leukemias, chronic and acute myeloid leukemias, mastocytomas, and multiple myeloma. For example, anti-CD20-equivalent antibody components and immunoconjugates can be used to treat both indolent and aggressive forms of non-Hodgkin's lymphoma. The therapeutic compositions are also particularly useful for treatment of autoimmune disorders, such as immune-mediated autoimmune hemolytic anemia, canine granulomatous meningoencephalitis, rheumatoid arthritis, chronic superficial keratitis, systemic lupus erythematosus, bullous pemphigoid, pemphigus, and thrombocytopenias.

[00110] A radiolabeled antibody, immunoconjugate or fusion protein may comprise an α-emitting radioisotope, a β-emitting radioisotope, an Auger electron emitter, or a neutron capturing agent that emits α-particles or a radioisotope that decays by electron capture. Suitable radioisotopes include ^{198}Au, ^{32}P, ^{125}I, ^{131}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{67}Cu, ^{211}At, ^{213}Bi, ^{111}In, ^{67}Ga, ^{177}Lu, and ^{225}Ac, and the like.

[00111] As discussed above, a radioisotope can be attached to an antibody component directly or indirectly, via a chelating agent. For example, ^{67}Cu, considered one of the more promising radioisotopes for radioimmunotherapy due to its 61.5 hour half-life and abundant supply of

beta particles and gamma rays, can be conjugated to an antibody component using the chelating agent, p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid (TETA). Chase, "Medical Applications of Radioisotopes," in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Gennaro *et al.* (eds.), pages 624-652 (Mack Publishing Co. 1990). Alternatively, ^{90}Y, which emits an energetic beta particle, can be coupled to an antibody component using diethylenetriaminepentaacetic acid (DTPA). Moreover, a method for the direct radiolabeling of the antibody component with ^{131}I is described by Stein *et al., Antibody Immunoconj. Radiopharm. 4*: 703 (1991).

[00112] Alternatively, boron addends such as carboranes can be attached to antibody components, as discussed above.

[00113] Preferred immunoconjugates and fusion proteins to be used in conjunction with a naked antibody or an immunoconjugate or fusion protein that includes a drug, toxin or therapeutic radionuclide include conjugates of an antibody component and an immunomodulator. As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, such as tumor necrosis factor (TNF), and hematopoietic factors, such as interleukins (*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-10 and IL-12), colony stimulating factors (*e.g.*, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (*e.g.*, interferons-α, -β and -γ), the stem cell growth factor designated "S1 factor," erythropoietin and thrombopoietin. Examples of suitable immunomodulator moieties include IL-2, IL-6, IL-10, IL-12, IL-18, interferon-γ, TNF-α, and the like.

[00114] Subjects also can receive naked antibodies, immunoconjugates and fusion proteins with a separately administered cytokine, which can be administered before, concurrently or after administration of the naked antibodies, immunoconujugates or fusion protein. The cytokines enhance the activity of ADCC/NK, the effector cells that effect kill of tumor cells by binding to the Fc domain of human IgG1 antibodies, a domain that is present in C2B8 (CD20 antibody).

[00115] Antibody-immunomodulator immunoconjugates and antibody-immunomodulator fusion proteins provide a means to deliver an immunomodulator to a target cell and are particularly useful against tumor cells. The cytotoxic effects of immunomodulators are well known to those of skill in the art. See, for example, Klegerman *et al.*, "Lymphokines and

Monokines," in BIOTECHNOLOGY AND PHARMACY, Pessuto *et al.* (eds.), pages 53-70 (Chapman & Hall 1993). As an illustration, interferons can inhibit cell proliferation by inducing increased expression of class I histocompatibility antigens on the surface of various cells and thus, enhance the rate of destruction of cells by cytotoxic T lymphocytes. Furthermore, tumor necrosis factors, such as TNF-α, are believed to produce cytotoxic effects by inducing DNA fragmentation.

[00116] Useful cancer chemotherapeutic drugs for the preparation of immunoconjugates and fusion proteins include nitrogen mustards, camptothecins, doxorubicin and analogs, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, pyrimidine analogs, purine analogs, antibiotics, epipodophyllotoxins, platinum coordination complexes, hormones, and the like. Suitable chemotherapeutic agents are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19th Ed. (Mack Publishing Co. 1995), and in GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed. (MacMillan Publishing Co. 1985). Other suitable chemotherapeutic agents, such as experimental drugs, are known to those of skill in the art.

[00117] In addition, therapeutically useful immunoconjugates can be obtained by conjugating photoactive agents or dyes to an antibody composite. Fluorescent and other chromogens, or dyes, such as porphyrins sensitive to visible light, have been used to detect and to treat lesions by directing the suitable light to the lesion. In therapy, this has been termed photoradiation, phototherapy, or photodynamic therapy (Jori *et al.* (eds.), PHOTODYNAMIC THERAPY OF TUMORS AND OTHER DISEASES (Libreria Progetto 1985); van den Bergh, *Chem. Britain 22*:430 (1986)). Moreover, monoclonal antibodies have been coupled with photoactivated dyes for achieving phototherapy. Mew *et al., J. Immunol.* 130:1473 (1983); *idem., Cancer Res.* 45:4380 (1985); Oseroff *et al., Proc. Natl. Acad. Sci. USA* 83:8744 (1986); *idem., Photochem. Photobiol.* 46:83 (1987); Hasan *et al., Prog. Clin. Biol. Res.* 288:471 (1989); Tatsuta *et al., Lasers Surg. Med.* 9:422 (1989); Pelegrin *et al., Cancer* 67:2529 (1991). However, these earlier studies did not include use of endoscopic therapy applications, especially with the use of antibody fragments or subfragments. Thus, the present invention contemplates the therapeutic use of immunoconjugates comprising photoactive agents or dyes.

[00118] Multimodal therapies of the present invention further include immunotherapy with naked anti-CD20 and naked anti-CD19 antibodies supplemented with administration of anti-

CD21 and anti-CD22 antibodies, respectively, as well as with the co-administration of anti-HLA-DR, CD52 and/or CD74 antibodies in the form of naked antibodies or as therapeutic immunoconjugates. Anti-CD19 and anti-CD20 antibodies are known to those of skill in the art. See, for example, Ghetie *et al., Cancer Res.* 48:2610 (1988); Hekman *et al., Cancer Immunol. Immunother.* 32:364 (1991); Kaminski *et al., N. Engl. J. Med.* 329:459 (1993); Press *et al., N. Engl. J. Med.* 329:1219 (1993); Maloney *et al., Blood* 84:2457 (1994); Press *et al., Lancet* 346:336 (1995); Longo, *Curr. Opin. Oncol.* 8:353 (1996).

[00119] In another form of multimodal therapy, subjects receive naked antibodies, and/or immunoconjugates or fusion proteins, in conjunction with standard cancer chemotherapy. For example, "CVB" (1.5 g/m^2 cyclophosphamide, 200-400 mg/m^2 etoposide, and 150-200 mg/m^2 carmustine) is a regimen used to treat non-Hodgkin's lymphoma. Patti *et al., Eur. J. Haematol.* 51:18 (1993). Other suitable combination chemotherapeutic regimens are well known to those of skill in the art. See, for example, Freedman *et al.*, "Non-Hodgkin's Lymphomas," in CANCER MEDICINE, VOLUME 2, 3rd Edition, Holland *et al.* (eds.), pages 2028-2068 (Lea & Febiger 1993). As an illustration, first generation chemotherapeutic regimens for treatment of intermediate-grade non-Hodgkin's lymphoma include C-MOPP (cyclophosphamide, vincristine, procarbazine and prednisone) and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone). A useful second-generation chemotherapeutic regimen is m-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone and leucovorin), while a suitable third generation regimen is MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin and leucovorin). Additional useful drugs include phenyl butyrate and bryostatin-1. In a preferred multimodal therapy, both chemotherapeutic drugs and cytokines are co-administered with a naked antibody, immunoconjugate or fusion protein according to the present invention. The cytokines, chemotherapeutic drugs and antibody, immunoconjugate or fusion protein can be administered in any order, or together.

[00120] Subjects being treated for autoimmune diseases also may receive a multimodal therapy. In this case, naked antibodies, immunoconjugates and/or fusion proteins are administered in conjunction with standard agents used to treat autoimmune diseases. Multimodal therapy of Class III autoimmune diseases may comprise co-administration of therapeutics that are targeted against T-cells, plasma cells or macrophages, such as antibodies directed against T-cell epitopes, more particularly against the CD4 and CD5 epitopes.

Gamma globulins also may be co-administered. In some cases, it may be desirable to co-administer immunosupproessive drugs such as corticosteroids and possibly also cytotoxic drugs. In this case, lower doses of the corticosteroids and cytotoxic drugs can be used as compared to the doses used in conventional therapies, thereby reducing the negative side effects of these therapeutics. The supplemental therapeutic compositions can be administered before, concurrently or after administration of the naked antibodies, immunoconjugates and/or fusion proteins.

[00121] Drugs which are known to act on B-cells, myeloid cells, mast cells, plasma cells and/or T-cells are particularly useful in accordance with the present invention, whether conjugated to a targeting antibody, or administered as a separate component in combination with naked antibodies, immunoconjugates and/or fusion proteins. These include methotrexate, phenyl butyrate, bryostatin, cyclophosphamide, etoposide, bleomycin, doxorubicin, carmustine, vincristine, procarbazine, dexamethasone, leucovorin, prednisone, maytansinoids such as DM1, calicheamicin, rapamycin, leflunomide, FK506, immuran, fludarabine, azathiopine, mycophenolate, and cyclosporin. Drugs such as immuran, methotrexate, and fludarabine, which act on both B-cells and T-cells, are particularly preferred. Illustrative of toxins which are suitably employed in accordance with the present invention are ricin, abrin, ribonuclease, DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, *Pseudomonas* exotoxin, *Pseudomonas* endotoxin and RNases, such as onconase. See, for example, Pastan *et al.*, *Cell* 47:641 (1986), and Goldenberg, *CA - A Cancer Journal for Clinicians* 44:43 (1994). Other suitable drugs and toxins are known to those of skill in the art.

[00122] In general, the dosage of administered naked antibodies, antibody components, immunoconjugates, and fusion proteins will vary depending upon such factors as the patient's age, species, breed, weight, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of naked antibody, immunoconjugate or fusion protein which is in the range of from about 1 to 20, preferably 1 to 10, mg/kg. The naked antibodies are administered parenterally, and may given once, or more preferably, repeatedly. In a preferred embodiment, the animal initially is treated repeatedly over the course of several weeks. After the initial regimen of repeated treatments, the animal may be placed on a maintenance regimen which involves treatments on a monthly, bimonthly or quarterly basis, as needed, for example.

[00123] Administration of antibody components, immunoconjugates or fusion proteins to a patient can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses.

[00124] Those of skill in the art are aware that intravenous injection provides a useful mode of administration due to the thoroughness of the circulation in rapidly distributing antibodies. Intravenous administration, however, is subject to limitation by a vascular barrier comprising endothelial cells of the vasculature and the subendothelial matrix. Still, the vascular barrier is a more notable problem for the uptake of therapeutic antibodies by solid tumors. Lymphomas have relatively high blood flow rates, contributing to effective antibody delivery. Intralymphatic routes of administration, such as subcutaneous or intramuscular injection, or by catheterization of lymphatic vessels, also provide a useful means of treating lymphomas.

[00125] As described above, the present invention contemplates therapeutic methods in which naked antibody components are supplemented with immunoconjugate or fusion protein administration. In one variation, naked antibodies are administered with low-dose radiolabeled antibodies or fragments or fusion proteins. As a second alternative, naked antibodies are administered with cytokine immunoconjugates. As a third alternative, naked antibodies are administered with immunoconjugates comprising a drug or toxin as a therapeutic agent. As a fourth alternative, naked antibodies are administered in conjunction with cytokine or chemotherapeutic agents that are not conjugated to any antibody component.

[00126] For an ^{131}I-labeled immunoconjugate, a preferable dosage is in the range of 0.25 to 2 mCi/kg, which can be repeated. In contrast, a preferred dosage of ^{90}Y-labeled immunoconjugates is in the range from 0.1 to 0.6 mCi/kg, which also can be repeated.

[00127] Where the targeted B-cell antigen is the CD20 antigen, the preferred antibody component is an antibody derived from a B1 antibody, including murine B1 monoclonal antibody, chimeric C2B8 antibody (rituximab), and humanized or caninized CD20 antibody.

[00128] Immunoconjugates having a boron addend-loaded carrier for thermal neutron activation therapy will normally be effected in similar ways. However, it will be advantageous to wait until non-targeted immunoconjugate clears before neutron irradiation is

performed. Clearance can be accelerated using an antibody that binds to the immunoconjugate. See U.S. patent No. 4,624,846 for a description of this general principle.

[00129] The naked antibodies, immunoconjugates and fusion proteins alone, or conjugated to liposomes, can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, REMINGTON'S PHARMACEUTICAL SCIENCES, 19th Ed. (1995).

[00130] For purposes of therapy, naked antibodies, immunoconjugates and/or fusion proteins are administered to a patient in a pharmaceutically acceptable carrier in a therapeutically effective amount. A "therapeutically effective amount" is an amount that is physiologically significant. An amount is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. In the present context, an amount is physiologically significant if its presence results in the inhibition of proliferation, inactivation, or killing of targeted cells.

[00131] Additional pharmaceutical methods may be employed to control the duration of action of a naked antibody, immunoconjugate or fusion protein in a therapeutic application. Control release preparations can be prepared through the use of polymers to complex or adsorb the antibody component, immunoconjugate or fusion protein. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood *et al.*, *Bio/Technology* 10:1446 (1992). The rate of release of an antibody component (or immunoconjugate) from such a matrix depends upon the molecular weight of the protein, the amount of antibody component/immunoconjugate/fusion protein within the matrix, and the size of dispersed particles. Saltzman *et al.*, *Biophys. J.* 55:163 (1989); Sherwood *et al.*, *supra*. Other solid dosage forms are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 19th ed. (1995).

[00132] The present invention also contemplates a method of treatment in which immunomodulators are administered to prevent, mitigate or reverse radiation-induced or

drug-induced toxicity of normal cells, and especially hematopoietic cells. Adjunct immunomodulator therapy allows the administration of higher doses of cytotoxic agents due to increased tolerance of the recipient mammal. Moreover, adjunct immunomodulator therapy can prevent, palliate, or reverse dose-limiting marrow toxicity. Examples of suitable immunomodulators for adjunct therapy include G-CSF, GM-CSF, thrombopoietin, IL-1, IL-3, IL-12, and the like. The method of adjunct immunomodulator therapy is disclosed by Goldenberg, U.S. patent No. 5,120,525.

[00133] For example, recombinant IL-2 may be administered intravenously as a bolus at 6×10^5 IU/kg or as a continuous infusion at a dose of 18×10^6 IU/m^2/d. Weiss *et al., J. Clin. Oncol. 10*:275 (1992). Alternatively, recombinant IL-2 may be administered subcutaneously at a dose of 12×10^6 IU. Vogelzang *et al., J. Clin. Oncol.* 11:1809 (1993). Moreover, INF-γ may be administered subcutaneously at a dose of 1.5×10^6 U. Lienard *et al., J. Clin. Oncol.* 10:52 (1992). Furthermore, Nadeau *et al., J. Pharmacol. Exp. Ther.* 274:78 (1995), have shown that a single intravenous dose of recombinant IL-12 (42.5 μg/kilogram) elevated IFN-γ levels in rhesus monkeys.

[00134] Suitable IL-2 formulations include PROLEUKIN (Chiron Corp./Cetus Oncology Corp.; Emeryville, CA) and TECELEUKIN (Hoffmann-La Roche, Inc.; Nutley, NJ). ACTIMMUNE (Genentech, Inc.; South San Francisco, CA) is a suitable INF-γ preparation.

[00135] The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

[00136] **EXAMPLE 1**

[00137] Peripheral blood was collected from a dog to test crossreactivity with dog lymphocytes. Murine MAbs against a panel of human leukocyte antigens were included in the assay. A human blood sample was tested at the same time as a control. Single color indirect flow cytometry analysis was performed.

[00138] Reactivity of the MAb panel with the human lymphocytes was within the expected range. Mabs 1F5 (anti-CD20), an anti-CD20 MAb from Biogenex, and L243 (MHC class II) all reacted with the dog lymphocytes. LL2, LL1, A103, Leu 4 (Becton-Dickinson anti-CD3),

Leu-16 (Becton-Dickinson anti-CD20) and H-Le-1 (Becton-Dickinson anti-CD45) did not show high crossreactivity with the dog lymphocytes.

	Human Lymphocytes	Canine Lymphocytes
Mab	**%Positive**	**%Positive**
Ag8	3.20	1.19
A103	33.75	2.91
LL2	10.19	3.48
LL1	10.06	0.62
Leu-4 (CD3, T-cells)	70.60	0.44
1F5 (CD20, B-cells)	11.29	24.70
Biogenex (CD20, B-cells)	6.64	23.48
Leu-16 (CD20, B-cells)	9.15	3.17
H-Le-1 (CD45, all WBCs)	95.91	1.77
L243 (MHC class II)	16.80	79.12

[00139] **EXAMPLE 2**

[00140] Treatment of a Dog with Aggressive Non-Hodgkin's B-cell Lymphoma in Lymph Nodes and Bone Marrow

[00141] A 65-pound, 7-year old male Golden Retriever is diagnosed with diffuse large cell aggressive lymphoma. The dog is placed on combination chemotherapy with vincristine, cyclophosphamide, prednisolone, and doxorubicin, with good response. However, the dog subsequently is characterized as having progressive lymphadenopathy, and seven months after this is found to have extensive lymphoma infiltration of bone marrow, extensive lymphoadenopathy of neck, chest, abdomen, pelvis, and hepatosplenomegaly (Day 0).

[00142] The dog is given therapy with 1F5 monoclonal antibody. The dog is infused intravenously with 120 mg of 1F5 antibody, and the treatment is repeated weekly for 4 weeks following this initial treatment. Four months after the final dose of 1F5, a computerized tomography scan of the patient shows no evidence of lymphoma, and all signs and symptoms of the disease were not evident.

[00143] **EXAMPLE 3**

[00144] Treatment of a Dog with Relapsed Intermediate-Grade Non-Hodgkin's Lymphoma

[00145] A 78-pound, 9-year old, German Shepherd Dog with intermediate grade non-Hodgkin's lymphoma receives chemotherapy, which initially leads to a complete remission for five months, followed by another course of chemotherapy which results in stable disease

for six months. The dog then presents with recurrent lymphoma in the chest and in a neck lymph node, both measurable by computerized tomography and palpation, respectively.

[00146] The patient is infused with a ^{90}Y-labeled immunoconjugate of L243 monoclonal antibody weekly for two weeks, at a radiation dose of 8 mCi in 50 mg of antibody protein. Three weeks later, palpation of the neck node enlargement shows a measurable decrease, while a repeat computerized tomography scan of the chest shows a marked reduction in tumor. Follow-up measurements made at ten weeks post therapy show evidence of the disease in the neck or the chest being reduced by a about 60 percent. Since new disease is not detected elsewhere, the patient is considered to be in partial remission. Follow-up studies every 10-12 weeks confirms a partial remission for at least 7 months post therapy.

[00147] **EXAMPLE 4**

[00148] Treatment of a Cat with Relapsed Lymphoma

[00149] A 10-pound, 12-year-old, domestic short hair presents with enlargement of a single submandibular lymph node. After excision, there is recurrence of the lesion at 6 months. The lesion is again excised, but then reappears 6 months later. The cat is then given weekly treatments for 4 weeks with an ^{131}I-labeled immunoconjugate of anti-CD20 B1 monoclonal antibody, at a radiation dose of 15 mCi in 45 mg antibody protein. The treatment is repeated 3 months later. When examined 3 months after the last treatment, a marked decrease can be palpated. No recurrence of the disease is observed for over one year.

[00150] **EXAMPLE 5**

[00151] Combination Therapy of a Dog with Autoimmune Hemolytic Anemia

[00152] A 35-pound cocker spaniel presents with immune-mediated autoimmune hemolytic anemia. The dog is given weekly treatments with a combination of 1F5 and L243, and is given 10 mg prednisone twice a day. Red blood cell counts done following treatment reveal values within normal ranges, and other abnormal signs and symptoms disappear for 2 months.

[00153] **EXAMPLE 6**

[00154] Combination Therapy of a Dog with Myeloid Leukemia

[00155] A 25-pound, 9-year-old female Beagle is diagnosed with a chronic myeloid leukemia. The dog is placed on combination chemotherapy with vincristine, cyclophosphamide, and

doxorubicin. After an initial period of remission, the disease recurs, and the dog is again treated with vincristine, cyclophosphamide, and doxorubicin, but additionally is given 50 mg of CD33 (M195) monoclonal antibody. The dog is infused intravenously, and the treatment is repeated weekly for 4 weeks following this initial treatment. Three months after the final treatment, a peripheral blood cell count, as well as a bone marrow biopsy, show evidence of a reduction of the leukemic cells by about 75 percent.

[00156] Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

[00157] All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.